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Combination treatment of PKD utilizing dual inhibition of EGF-receptor activity and ligand bioavailability

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Combination treatment of PKD utilizing dual inhibition of EGF-receptor activity and ligand bioavailability.

Background. We have previously demonstrated an essential role for increased epidermal growth factor receptor (EGFR) activity in mediating renal cyst formation and biliary ductal ectasia (BDE) in murine models of autosomal-recessive polycystic kidney disease (ARPKD) such as the BPK mouse. The current study was designed to determine (*1*) if treatment with a second-generation inhibitor of EGFR tyrosine kinase activity, EKB-569, was effective in treatment of ARPKD; (*2*) if tyrosine kinase inhibitor therapy used in combination with pharmacologic reduction of the availability of transforming growth factor-α (TGF-α), using WTACE2, could provide improved therapeutic efficacy and/or decrease potential toxicity; and (*3*) if effectiveness of treatment could be monitored noninvasively in murine ARPKD models by use of serial ultrasonography.

Methods. BPK litters were treated with EKB-569 by intraperitoneal injection from postnatal day 7 to postnatal day 21. EKB-569's effectiveness alone or in combination with WTACE2 was measured by reduction in kidney weight/body weight ratios, morphometric renal cystic index, and evaluation of renal function. Renal ultrasound was performed on normal and cystic animals, under different therapeutic regimens, utilizing a 15 mHz linear array transducer, and ultrasound data were compared with histology and renal functional data.

Results. Treatment of BPK mice with EKB-569 alone resulted in a marked reduction of kidney weight/body weight ratios, dramatically reduced collecting tubule cystic index, as well as BDE, and improved renal function. The combined treatment with EKB-569 and WTACE2 permitted a 67% reduction in EKB-569 dosage necessary to achieve results equivalent to those produced with EKB-569 alone. Untreated cystic animals died of renal failure, on average, at postnatal day 24 with a collecting tubule cystic index of 4.8, significant BDE, and maximal urine osmolarity of 361 mOsm. Cystic animals treated with EKB-569 and WTACE2 to postnatal day 21 were alive and well with normal renal function, a reduced collecting tubule cystic index of 1.7 ($P < 0.02$), improvement in BDE, and a threefold increase in maximum urinary concentrating ability ($P < 0.01$). Renal ultrasound could reliably detect cystic kidneys as early as postnatal day 7 and the natural history as well as effects of therapeutic intervention were clearly delineated by ultrasound evaluation.

Conclusion. This study demonstrates that in murine ARPKD (*1*) EKB-569 is as effective as first-generation EGFR tyrosine kinase inhibitors in reducing cyst formation and preserving renal function; (*2*) combination therapy with EKB-569 and WTACE2 provides maximum efficacy in improving renal and biliary abnormalities, at lower doses, thereby minimizing potential toxicity; and (*3*) renal ultrasound provides a simple, reliable, noninvasive method of following natural history and effect of treatment regimens.

Key words: tyrosine kinase inhibition, epidermal growth factor receptor, autosomal-recessive polycystic kidney disease, *bpk* mice, collecting tubule cysts.

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Polycystic kidney disease (PKD), a leading cause of end-stage renal disease, encompasses two genetically distinct conditions: autosomal-dominant polycystic kidney disease (ADPKD) and autosomal-recessive polycystic kidney disease (ARPKD) [1]. ADPKD occurs in 1:300 to 1:1000 individuals and most, if not all, disease is caused by mutations in one of two known genes, *PKD1* or *PKD2* [2, 3]. ADPKD is characterized by renal cyst formation in all tubular segments and extrarenal manifestations, including hepatic and pancreatic cysts, cerebral aneurysms, and an increased incidence of diverticulosis and valvular heart abnormalities [4]. ARPKD occurs less frequently, in approximately 1:20,000 live births, and all clinical cases appear to result from mutations in a single, recently identified gene, *PKHD1* [5, 6]. ARPKD is invariably characterized by the formation and enlargement of renal collecting tubule cysts as well as biliary ductal plate abnormalities [biliary ductal ectasia (BDE)] and hepatic fibrosis [7].

Cystic renal epithelia share common phenotypic abnormalities despite the different genetic mutations that underlie the disease. Numerous animal models as well as in vitro cell culture systems utilizing human and animal kidney cells have been used to delineate the cellular

pathophysiology of cystogenesis. These studies have reported abnormalities in fluid secretion, extracellular matrix composition, differentiation, and focal epithelial cellular proliferation [8]. Evidence from a number of laboratories demonstrates a significant role for the epidermal growth factor receptor (EGFR) axis in promoting epithelial hyperplasia in cystic epithelia, with resultant renal cyst formation and enlargement in both murine and human ADPKD and ARPKD [9–19]. In addition, evidence from murine models suggests that similar abnormalities of the EGFR axis may mediate biliary epithelial hyperplasia and BDE [13, 14, 20].

We have previously identified a central role for increased EGFR tyrosine kinase activity in promoting renal cyst formation and progressive enlargement by demonstrating a direct correlation between reduced cyst formation and decreased EGFR activity (i.e., autophosphorylation). Reduction of EGFR activity was accomplished, in vivo, by (*1*) genetic manipulation [18]; (*2*) pharmacologic inhibition of receptor activation with EKI-785 (EKI), an agent that covalently binds to the EGFR adenosine triphosphate (ATP) binding site [20, 21]; and (*3*) pharmacologic reduction of the availability of a cystogenic EGFR ligand, transforming growth factor-alpha (TGF-α) with a tumor necrosis factor-α (TNF-α) converting enzyme inhibitor, WTACE2 [22]. The purpose of the current study was to determine if a second-generation inhibitor of EGFR tyrosine kinase activity, EKB-569 (EKB) [23], was (*1*) as effective as EKI in reducing the development and enlargement of collecting tubule cysts and BDE in a well-characterized murine model of ARPKD; and (*2*) could be used in combination with WTACE2 to improve therapeutic response or permit reduction in doses of EKB while maintaining effective tyrosine kinase activity reduction. In addition, the feasibility of using renal ultrasound to identify cystic kidneys at an early stage of renal cyst formation and to utilize sonography as a noninvasive method to monitor the natural history of disease as well as therapeutic effectiveness of specific therapies was evaluated.

Results demonstrate that EKB was as effective as EKI in retarding in vivo renal cyst formation and BDE in BPK cystic mice, resulting in a marked reduction in the number and size of collecting tubule cystic lesions and improved renal survival and tubular function. Pharmacologic inhibition of EGFR activity with EKB used in combination with WTACE2 permitted a 67% reduction in EKB dosage necessary to maximally effect collecting tubule cyst reduction, preserve renal function, and minimize BDE. In addition, renal ultrasound provided a reliable, noninvasive, means of monitoring the natural history and effectiveness of experimental therapeutic regimens in vivo.

METHODS

EGFR tyrosine kinase inhibition

Tyrosine kinase inhibition was accomplished with EKB, a derivative of EKI, with improved pharmacokinetics and enteral bioavailability compared to the parent compound, EKI [23]. EKB covalently binds to EGFR, inhibits kinase activity of the protein (IC_{50} = 38.5 nmol/L), blocks EGF-stimulated autophosphorylation of the receptor without changing the total amount of EGFR protein, inhibits cell proliferation, and blocks the growth of tumors that overexpress EGFR [23]. The duration of EKB activity is dependent upon the half-life of the compound as well as the turnover rate of EKB-bound EGFR in the plasma membrane. Preliminary time course and dose response experiments demonstrated that intraperitoneal administration of EKB every 3 days resulted in optimal receptor inhibition with minimal toxicity.

Reduction of ligand availability

TGF-α is produced as a prepropeptide that undergoes proteolytic cleavage at the cell membrane to produce the mature, secreted moiety [24]. The cleavage of the prepropeptide is mediated by TNF-α converting enzyme (also known as Adam 17) [25]. Reduction of soluble TGF-α was accomplished with WTACE2, a novel metalloproteinase inhibitor specific for TNF-α converting enzyme developed by Wyeth-Ayerst Research (Pearl River, NJ, USA). WTACE2 is an orally active, broad-spectrum inhibitor of matrix metalloproteinase (MMP) with nanomolar activity against MMP-1, MMP-9, and MMP-13 and little or no toxicity demonstrated at doses of 250 mg/kg per day [22].

The Balb/c-bpk/bpk (BPK) model

The BPK model, a murine model of ARPKD, arose as a spontaneous mutation in an inbred colony of Balb/c mice and has been extensively characterized [19]. Homozygous BPK mice, *bpk*/*bpk,* mimic human ARPKD, including the development of large collecting tubule cysts, and BDE. Affected animals develop massively enlarged kidneys and die of renal failure at an average postnatal age of 24 days [13, 14, 20]. Due to the recessive nature of this disease, wild-type+/+ and heterozygous *bpk*/+ mice are phenotypically normal.

Dose response studies

Entire litters from proven BPK heterozygous breeders (including BPK cystic *bpk/bpk*, heterozygous *bpk/+*, and wild-type+/+ pups) received EKB at 30, 60, or 90 mg/kg, by intraperitoneal injections every 3 days starting at postnatal day 7. These dosages were based on previous pharmacologic studies of EKI in the BPK mouse [26] and published pharmacokinetic data comparing EKI and EKB effectiveness [23]. Morphologic analysis was per-

formed to determine reduction of renal cystic lesions as well as biliary proliferation and BDE and evaluation of renal and extrarenal organ toxicity. Animals were treated from postnatal day 7 to postnatal day 19 (five doses), and kidney, liver, heart, spleen, stomach and thymus tissues were harvested at postnatal day 21. A minimum of six affected animals and 10 unaffected animals were analyzed at each dosage. Control animals for these studies included EKI treated and untreated (vehicle only) phenotypically normal littermates. The vehicle used for intraperitoneal injections was 2% Tween 80, 0.5% methylcellulose in water.

Combination of EKB-569 and WTACE2

Previous studies demonstrated that maximum cyst reduction, in the absence of any toxicity, was achieved by WTACE2 administered at 100 mg/kg, intraperitoneally, daily [22]. WTACE2 was administered intraperitoneally at this dose from postnatal day 8 to postnatal day 20, on days between EKB-569 treatments (nine total doses).

Histology, immunohistology, and determination of segmental nephron cyst localization

All kidney and liver tissues were fixed in 4.0% paraformaldehyde in phosphate buffer (pH 7.4) for 30 minutes at 4°C. Tissues were then washed, dehydrated through graded acetone, and embedded in Immunobed™ plastic embedding medium (Polysciences, Warrington, PA, USA). Sections were cut at 4 μm on an ultramicrotome, mounted on glass slides, and stained with hematoxylin (all tissues) or segment-specific lectins (kidney only). Segmental nephron cystic localization and collecting tubule cystic index were quantitated in each experimental group by combining morphometric analysis with light microscopy and immunohistologic techniques [17–20, 27]. Cyst localization was studied by segment-specific lectin binding using *Dolichos biflorus* agglutinin (DBA) as a marker for collecting tubules and *Lotus tetragonolobus* (LTA) as a marker for proximal tubules [13, 17–20, 28]. In liver tissue, BDE was assessed morphologically by previously published methods [20].

The immunostaining procedure used was our previously described postembedded staining technique specifically developed for localization of antigens and lectins in plastic sections [29]. Sections 4 μm thick were etched, trypsinized and incubated overnight at 4°C with biotinylated lectins (3.57 ug/mL for LTA and 6.25 ug/mL for DBA), followed by incubation with extraviden peroxidase (1/400), or extraviden alkaline phosphatase (1/200), for 90 minutes at room temperature. Sections incubated with extraviden peroxidase were developed with 0.05% diaminobenzidine, 0.01% hydrogen peroxide for 10 minutes, and those stained with extraviden alkaline phosphatase were developed with Fast-Red. All sections were counterstained with hematoxylin.

Kidney weight to body weight ratio and renal cystic index

At postnatal day 21, total body weights of control and cystic, treated and untreated, animals were obtained along with the combined weight of both excised kidneys for the determination of kidney weight to body weight ratios. The degree of collecting tubule cyst formation or cystic index was quantitated by segment-specific morphometric analysis of cyst formation. The index was derived from basic light microscopic morphometric methods [27] and has been standardized to quantitate cyst formation in vivo and in vitro [13, 17, 18, 20, 22]. Following routine histologic preparation, 10 to12 evenly spaced, 4 μm thick sections of kidney were graded for cyst localization and severity of formation in collecting tubule tubular segments (DBA-positive) [20, 22]. For each treatment group, a collecting tubule cystic index was determined on a total of at least six affected pups.

Analysis of renal function and maximal urinary concentrating ability

Animals were deprived of water for 12 hours prior to collection of urine samples for urine osmolarity measurements. Blood samples were obtained by cardiac puncture for serum blood urea nitrogen (BUN) and creatinine measurements. Serum BUN was quantitatively determined on an automated clinical chemistry analyzer using the Boehringer Mannheim urease-triggered methodology based upon the method of Talke and Schubert [30]. Serum creatinine was quantitative determined on an automated clinical chemistry analyzer by the Seelig modification of the Jaffe method [31].

Hepatic BDE

Following routine histologic preparation, eight evenly spaced, at least 32 μm apart, 4 μm thick, hematoxylin-stained liver sections were graded (0 to 4) for BDE and biliary epithelial proliferation using previously published methods [20].

Ultrasonography

Renal ultrasound was performed using a 15 mHz linear array transducer. Control and cystic animals, ages postnatal day 7, postnatal day 14, and postnatal day 21, under normal conditions as well as pharmacologic regimens, were sedated and length and width of both right and left kidneys were measured. The accuracy of renal ultrasound was evaluated by comparing in vivo ultrasound measurements to postmortem measurements of excised kidneys from animals sacrificed immediately following in vivo renal ultrasound.

Statistical analysis

The significance of differences between experimental groups was determined by a one-way or two-way analysis

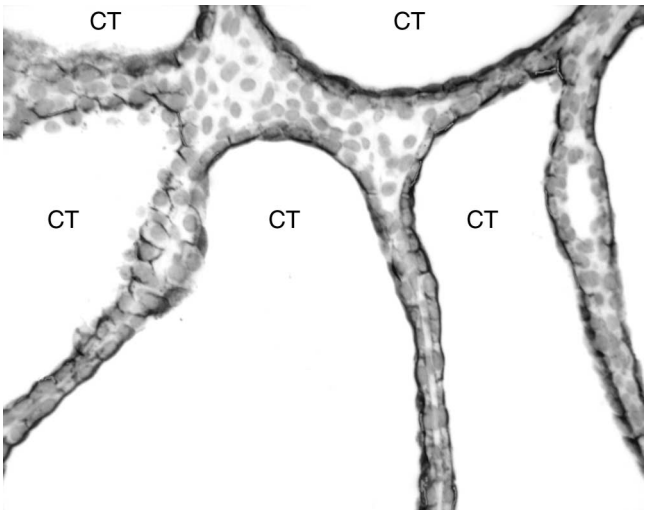

Fig. 1. Day 21 untreated cystic kidney. Collecting tubules (CT) labeled with lectin *Dolichos biflorus* agglutin (DBA). Photograph demonstrates very large collecting tubule cysts with little normal renal parenchyma, a typical characteristic of end-stage BPK cystic kidney (original magnification 100×).

of variance (ANOVA) with least significant difference comparisons of the means or Student *t* test as appropriate. [32]. Results are expressed as mean ± SD.

RESULTS

Dose response studies

Both normal and cystic mice animals were administered EKB, by intraperitoneal injections, at 30, 60 or 90 mg/kg, every 3 days starting at postnatal day 7. Kidney and liver tissues were harvested at postnatal day 21 and the following were assessed: kidney weight to body weight ratios, segment-specific morphometric analysis of a cystic index, serum BUN, creatinine, and maximum urinary concentrating ability. Figure 1 demonstrates the large collecting tubule cystic lesions (cystic index = 5) present in day 21 untreated cystic kidneys. All parameters improved in a dose dependent manner up to the maximum effective dose of 90 mg/kg, the same dose utilized in our previously published benchmark treatment protocol with EKI [20]. Figure 2 demonstrates that maximal reduction in collecting tubule cyst growth, without morphologic evidence of renal toxicity, required EKB administration at 90 mg/kg every 3 days. Table 1 demonstrates that compared to untreated controls ($N = 10$), animals treated with EKB (90 mg/kg) ($N = 6$) showed a 70% ± 10% ($P < 0.02$) reduction in kidney weight to body weight ratio, a cystic index reduction of 65% ± 10% ($P < 0.02$), and a significant reduction in BUN from 191 ± 2 to 28 ± 3 ($P < 0.02$). EKB treatment (90 mg/kg every 3 days) also resulted in a 300% reduction in creatinine and a 275% improvement in maximum urine osmolarity. These results are similar to those achieved with our previously published benchmark treatment trial with EKI (90 mg/kg every 3 days) (Fig. 2) (Table 1) [20].

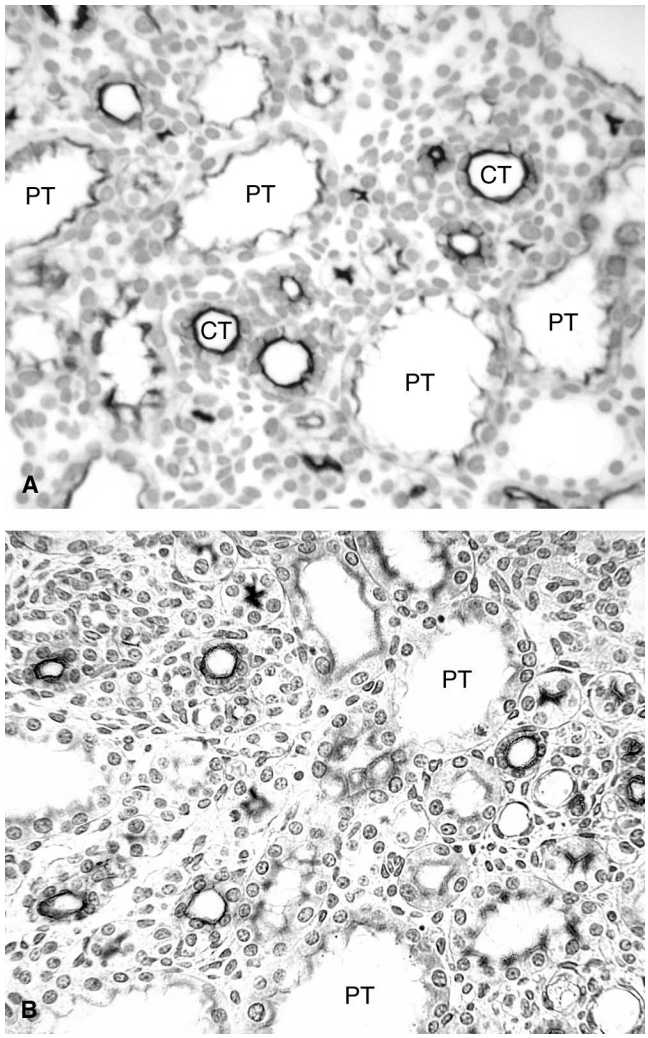

Fig. 2. Representative morphology of (*A*) EKB-569–treated kidney from postnatal day 21 BPK cystic animal and (*B*) EKI-785–treated kidney from postnatal day 21 BPK cystic animal. *Lotus tetragonolobus* (LTA)-labeled proximal tubule (PT), and *Dolichos biflorus* agglutin (DBA)-labeled collecting tubules as shown. Data demonstrate that EKB-569 treatment at 90 mg/kg every 3 days results in dramatic reduction in size and number of collecting tubule (CT) cystic lesions equivalent to the reduction of collecting tubule cyst growth with EKI-785 treatment [20] (original magnification 40×).

Treatment of unaffected mice with EKB from postnatal day 7 to postnatal day 21 (90 mg/kg every 3 days) resulted in a 7% decrease in total body weight and a 5% decrease in kidney weight (data not shown). The kidney weight to body weight ratio of unaffected animals remained unchanged at 1.2%. However, treatment of cystic animals with EKB resulted in a markedly reduced kidney weight to body weight ratio from 22% in untreated cystic mice to 6.6% in EKB-treated cystic mice (body weight reduced 5%, kidney weight reduced 70%). This reduction in kidney weight to body weight ratio of cystic EKB-treated animals demonstrates that the re-

Table 1. Kidney weight/body weight ratios, cystic index and renal function with EKI-785 or EKB-569 treatment

	Kidney weight/body weight %	Collecting tubule cystic index (CI)	BUN *mg/dL* CR *mg/dL*	Maximum urine osmolarity
Untreated cystic ($N = 10$)	22.1 ± 3	4.8 ± 0.4	191 ± 2 0.6 ± 0.2	361 ± 74
Cystic-treated EKI-785 (90 mg/kg) ($N = 14$)	5.6 ± 3[a]	1.6 ± 0.5[a]	19 ± 4[b] 0.2 ± 0.1[a]	1099 ± 47[b]
Cystic-treated EKB-569 (90 mg/kg) ($N = 6$)	6.6 ± 2[c]	1.7 ± 0.5[c]	28 ± 3[c] 0.2 ± 0.1[c]	994 ± 54[c]

Abbreviations are: BUN, blood urea nitrogen; CR, creatinine.
[a] EKI-785 vs. untreated $P < 0.02$
[b] EKI-785 vs. untreated $P < 0.01$
[c] EKB-569 vs. untreated $P < 0.02$
EKB-569 vs. EKI-785, NS

duced kidney size observed was not due to nonspecific, global effects on growth.

EKB and EKI treatment also altered the cyst localization profile of BPK mice. In BPK cystic mice [13], other murine models of ARPKD (cpk [33], orpk [34]), as well as human ARPKD [35], cystic lesions first appear in the proximal tubules. As the disease progresses, the site of cystic lesions shifts to the collecting tubule. Untreated BPK mice have an equal ratio of proximal tubule to collecting tubule cysts at day 10. The majority of cysts (85% to 90%) in affected mice at postnatal day 21 are localized to the collecting tubule (from inner medulla to cortex) and are much larger than those localized to proximal tubule segments. In EKB- and EKI-treated BPK postnatal day 21 cystic mice, the ratio of proximal tubule to collecting tubule cysts is nearly equal and the proximal tubule cysts are slightly larger than the collecting tubule cysts (Fig. 2).

Combination therapy (EKB-569 and WTACE2)

Normal and cystic mice received EKB, intraperitoneally, in doses ranging from 30 mg/kg to 60 mg/kg, every 3 days starting at postnatal day 7. WTACE2 was administered intraperitoneally on intervening days at 100 mg/kg. Figure 3A demonstrates that reduction of EKB to 30 mg/kg in combination with WTACE2 (100 mg/kg) provided maximum reduction in collecting tubule cyst formation, comparable to the morphologic improvement with EKB (90 mg/kg every 3 days) (Fig. 3B). As seen in Table 2, all parameters of cystic disease measured in this study showed comparable improvement with EKB alone (90 mg/kg every 3 days) or with the combination of EKB (30 mg/kg every 3 days) and WTACE2 (100 mg/kg) when compared to untreated cystic animals.

Figure 4 demonstrates that combination therapy resulted in reduction of BDE comparable to results previously achieved with EKI [20]. Treatment with 30 mg/kg EKB and 100 mg/kg WTACE resulted in a significantly ($P < 0.02$) decreased BDE index value of 1.4 (Fig. 4C) (Table 2) compared to the untreated control BDE index value of 3.2 (Fig. 4B) (Table 2).

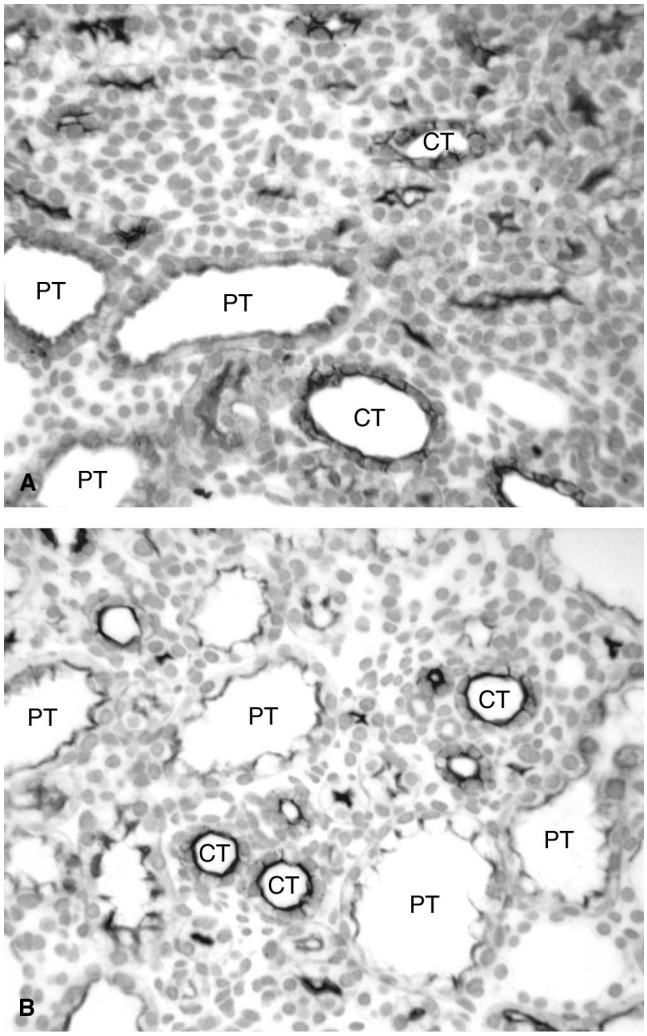

Fig. 3. Morphologic comparison of (*A*) EKB in combination with WTACE2 and (*B*) EKB alone, in postnatal day 21 BPK kidneys. Shown are *Lotus tetragonolobus* (LTA)-labeled proximal tubules (PT) and *Dolichos biflorus* agglutin (DBA)-labeled collecting tubules (CT). Data demonstrate that EKB-569 treatment (30 mg/kg every 3 days) in combination with WTACE2 (100 mg/kg on intervening days) results in dramatic reduction in size and number of collecting tubule cystic lesions equivalent to the reduction of collecting tubule cyst growth with EKB-569 (90 mg/kg every 3 days) alone. The dose of EKB-569 can be reduced by 67% when used in combination with WTACE2 (original magnification 40×).

Table 2. Comparison of kidney weight/body weight ratios, cystic index, and renal function with EKB-569 alone or in combination with WTACE2

	Kidney weight/body weight %	Collecting tubule cystic index (CI)	BUN *mg/dL*	CR *mg/dL*	Maximum urine osmolarity	BDE
Untreated-cystic ($N = 10$)	22.1 ± 3	4.8 ± 0.4	191 ± 2	0.6 ± 0.2	361 ± 74	3.2 ± 0.6
Cystic-treated EKB-569 (30 mg/kg + WTACE2) ($N = 8$)	6.8 ± 3[a]	1.8 ± 0.4[a]	24 ± 4[a]	0.2 ± 0.1[b]	958 ± 68[a]	1.4 ± 0.4[a]
Cystic-treated EKB-569 (90 mg/kg) ($N = 6$)	6.6 ± 2[a]	1.7 ± 0.5[a]	28 ± 3[a]	0.2 ± 0.1[b]	994 ± 54[a]	1.3 ± 0.5[a]

Abbreviations are: BUN, blood urea nitrogen; CR, creatinine; BDE, biliary ductal ectasia.
[a] EKB-569 vs. untreated $P < 0.02$
[b] EKB-569 + WTACE2 vs. untreated $P < 0.05$
EKB-569 vs. EKI-785, NS (compare to Table 1)

Fig. 4. Representative morphology of postnatal day 21 untreated liver from cystic *bpk/bpk* animal (*A*), postnatal day 21 normal liver from unaffected *bpk/+* animal (*B*), and postnatal day 21 EKI-785–treated liver from cystic *bpk/bpk* animal (*C*). Data demonstrate that (*1*) livers of cystic animals show extensive BDE (A) [compared to livers of unaffected animals (B)]; and (*2*) EKB treatment results in dramatic reduction of biliary ectasia (C) vs. untreated liver (A), without morphologic evidence of liver toxicity [original magnification (A) 20×, (B and C) 40×].

Table 3. Accuracy of ultrasound measurements vs. postmortem (length $\times 10^{-3}$ cm)

	Postnatal day 7 (normal)	Postnatal day 7 (cystic)	Postnatal day 14 (normal)	Postnatal day 14 (cystic)	Postnatal day 21 (normal)	Postnatal day 21 (cystic)
US	403 ± 10	636 ± 30	657 ± 30	982 ± 40	788 ± 30	1660 ± 30
CV %	2.48	4.72	4.60	4.10	3.81	1.81
PM	400 ± 10	650 ± 30	670 ± 20	1022 ± 25	784 ± 30	1840 ± 50
CV %	2.50	4.62	2.99	2.45	3.83	2.72
Accuracy %	99	98	98	96	99	94

Abbreviations are: US, ultrasound; CV, coefficient of variance; PM, postmortem measurement; accuracy, the correlation between US and PM measurements.

Toxicology

Figure 2A (EKB-treated kidney, postnatal day 21) and 2B (EKI-treated kidney, postnatal day 21) demonstrate no morphologic evidence of renal toxicity. The lack of renal toxicity at 90 mg/kg every 3 days is supported by the fact that serum BUN and creatinine levels as well as the maximum urinary concentrating ability of treated postnatal day 21 animals was not significantly different from untreated control values. Pilot toxicology studies on other organs demonstrated no compound-related deaths and no microscopic changes in heart, spleen, stomach, or thymus with 90 mg/kg or less of EKB administered intraperitoneally every 3 days.

When used in combination with WTACE 2, EKB at doses of 50 or 60 mg/kg caused an increased runting of the animals (12% or greater) without significant improvement in renal morphology, BDE, or renal function, when compared to the 30 mg/kg dose. Additionally, the combination of EKB at 60 mg/kg with WTACE2 at 100 mg/kg produced a 15% mortality rate when WTACE2 was administered daily between EKB administration. Eliminating the WTACE2 treatment on day 8 only prevented this mortality but still resulted in increased runting of both normal and cystic animals.

Renal ultrasound

As seen in Table 3 and Figure 5, renal ultrasound was effective in identifying cystic animals as early as postnatal day 7. Both width and length measurements of either right or left cystic kidneys correlated directly with morphologic assessment of disease progression with and without treatment (histology, kidney weight to body weight ratio, and cystic index). Table 3 compares in vivo ultrasound measurements to postmortem measurements of excised kidneys taken immediately after the renal ultrasound. As shown in Table 3, the accuracy of in vivo renal ultrasound lengths was 98% to 99% accurate for normal kidneys from postnatal day 7 to postnatal day 21. The accuracy of renal ultrasound for length declined from 99% at postnatal day 7 to 94% at postnatal day 21 when clearly identifying the margins of such large kidneys was more difficult by renal ultrasound.

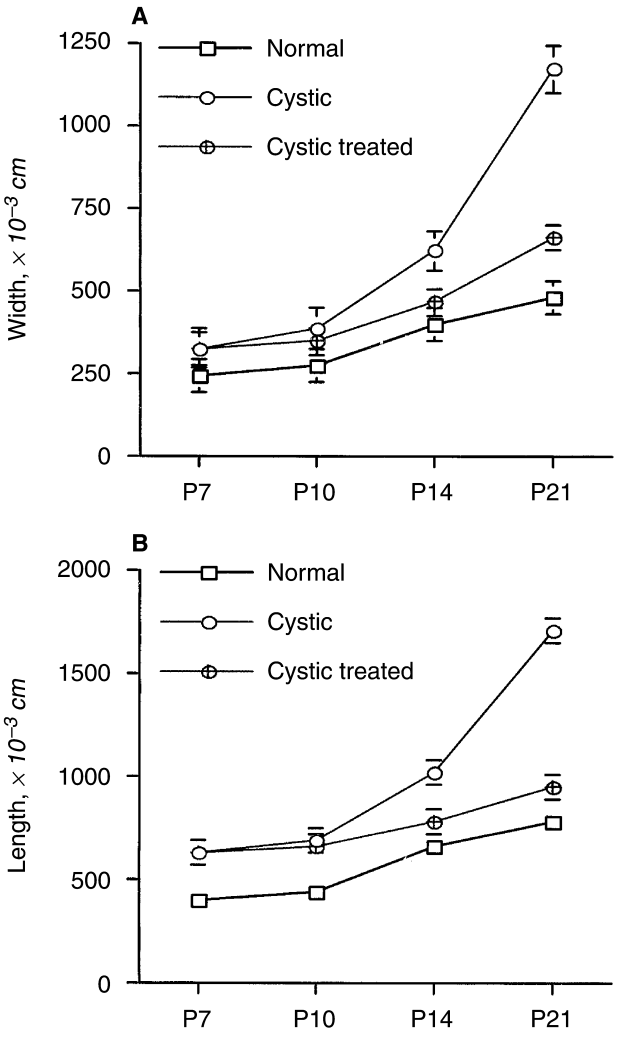

Fig. 5. Renal ultrasound measurements of (*A*) width and (*B*) length of kidney during natural disease progression and with combination treatment with EKB and WTACE2. Data demonstrate that renal ultrasound provides a noninvasive method of monitoring natural disease course and effectiveness of therapy. Combination treatment of nonaffected animals produced data that were identical to that of normal untreated control.

DISCUSSION

Numerous studies demonstrate that EGFR and its ligands such as TGF-α mediate disease progression in human and murine PKD (reviewed in [8]). Such studies demonstrate that (*1*) EGF and TGF-α are cystogenic in a variety of in vitro systems [15, 17, 26]; (*2*) cyst fluid from ADPKD, ARPKD, and rat and murine models of PKD contain immunoreactive EGF-like peptides in mitogenic quantities [11, 12]; (*3*) cystic kidneys have increased TGF-α mRNA levels [36]; (*4*) overexpression of TGF-α in transgenic mice causes renal cystic abnormalities [10]; (*5*) EGFR is overexpressed and mislocalized to the apical membrane of cystic epithelia in human ADPKD, human ARPKD, and murine models of ARPKD and ADPKD [9, 16, 19, 37, 38]; (*6*) abnormally expressed apical EGFR in PKD are capable of high-affinity EGF binding, autophosphorylation, and can initiate a signaling cascade that results in increased mitotic activity [19]; and (*7*) EGFR tyrosine kinase inhibitors decrease EGF-driven cyst progression in vitro [17, 26].

In previous studies, we have demonstrated the therapeutic effectiveness, in vivo, of EKI, an EGFR tyrosine kinase inhibitor, and WTACE2, an inhibitor of TGF-α processing in reducing progression of cystic disease in the BPK murine model of ARPKD [20, 22]. The different targets of these agents in the EGFR/TGF-α axis suggested the possibility that combination therapy might improve efficacy and/or decrease potential toxicity.

EKI has recently been replaced by a second-generation compound (EKB) that has improved pharmacokinetics and enteral bioavailability, compared to the parent compound [23].

The effectiveness of EKB was evaluated alone or in combination with WTACE2 in the BPK murine model of ARPKD. Specific outcomes evaluated included reduction of collecting tubule cyst formation and growth, BDE, and improvement in renal function. EKI is a second-generation member of a class of synthetic compounds designed to inhibit EGFR activity by covalently binding to the ATP binding site of EGFR [21, 23]. EKB inhibits (*1*) the catalytic activity of the purified EGFR kinase (IC_{50} = 420 pmol/L), (*2*) autophosphorylation of EGFR (IC_{50} = 5 nmol/L), and (*3*) mitogenesis in a variety of cells which overexpress EGFR [23].

In this study, normal and cystic BPK mice were administered EKB (30 to 90 mg/kg) by intraperitoneal injection every 3 days starting at day 7. Kidneys were harvested at day 21 and the following were assessed: kidney weight to body weight ratios, segment-specific morphometric assessment of cyst formation, serum BUN, creatinine, maximum urine osmolarity, and BDE. All parameters improved in a dose-dependent manner up to the maximum effective dose of 90 mg/kg. Compared to untreated controls (N = 10), treatment with EKB alone resulted in a 70% decrease in kidney weight to body weight ratio (P = 0.02), a 65% reduction in collecting tubule cystic index ($P < 0.02$), an 85% reduction in serum BUN ($P <$ 0.02), 300% reduction in creatinine, a 275% improvement in maximum urinary concentrating ability, and a 64% decrease in BDE.

Comparable improvements in all measures of cystic disease were achieved with a 67% reduction in EKB dosage when administered in combination with WTACE2. This study did not investigate lower doses of WTACE2 in combination with EKB-569 since previous dose response studies with WTACE2 [22] demonstrated that maximal effective dosage was 100 mg/kg daily in this model and that increasing WTACE2 doses to 250 mg/kg daily produced no toxicity.

The potential role of the EGFR axis in vascular and tubulointerstitial injury associated with renal and hepatic manifestations of PKD is a largely understudied area of great potential import. Evidence suggests that increased EGFR activity directly leads to growth of vascular smooth muscle cells [44], increased fibroblast-specific protein-1 (FSP-1) expression and increased extracellular matrix production [45]. All of these phenomena are associated with progressive fibrotic injury in PKD and other chronic progressive nephropathies [46]. Therefore, the inhibition of EGFR activity may provide dual therapeutic benefits by limiting tubular epithelial proliferation while reducing vascular and interstitial changes.

Additional studies are required to determine the precise window during which EGFR inhibition can be utilized safely to reduce cyst development and progressive enlargement, as well as the consequences of long-term therapy targeting the EGFR axis. The vital role of the EGFR axis in normal organogenesis seemingly precludes the use of this therapy during gestation or the early postpartum period. The use of EKB in combination with WTACE2 will hopefully provide the opportunity to maximize the efficacy to toxicity ratio by ultimately decreasing the amount of EKB required for chronic therapy in PKD.

The results of this study do not address the specific mechanisms by which a mutated murine or human ARPKD gene leads to qualitative and quantitative abnormalities in EGFR expression. The mutated genes responsible for murine models of PKD (CPK, BPK, and ORPK models) are on different chromosomes and none of these genes are syntenic with the human ARPKD or ADPKD genes. Increased apical collecting tubule EGFR expression demonstrated in both murine and human ARPKD and ADPKD is thus a common cellular phenotype downstream from a number of different primary gene defects in PKD [9, 13, 16, 18, 19, 34, 38]. A number of studies have demonstrated that this common cellular phenotype is maintained in primary and conditionally immortalized cystic epithelial in vitro [9, 19, 39]. This, along with the identification of *PKD1*, *PKD2*, and most

recently *PKHD1*, provide the opportunity to investigate the interactions between primary PKD mutations, EGFR mislocalization, and elucidation of pathogenic signaling pathways due to aberrant signaling from apical EGFR and mutated PKD proteins. Recent evidence demonstrates that both human and murine PKD proteins localize to primary cilia on the apical surface of all renal epithelial cells except intercalated cells, suggesting a role for primary cilia in PKD [40–43]. Although this hypothesis has yet to be proven, future discoveries regarding PKD protein interactions, and the normal and abnormal signaling pathways of PKD proteins may identify potential therapeutic targets downstream of the EGFR. However, inhibition of EGFR activity has proven to be the most effective in vivo treatment in murine PKD to date [47, 48].

We speculate that targeting the cystic EGFR cellular phenotype common to murine and human ARPKD and ADPKD with compounds such as EKB, in combination with inhibitors of ligand availability, such as WTACE 2, may have therapeutic value in the treatment of human PKD.

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